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First Detection of TR34 L98H and TR46 Y121F T289A Cyp51 Mutations in *Aspergillus fumigatus* Isolates in the United States

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Azole resistance in *Aspergillus fumigatus* is an increasing problem. The TR34 L98H and TR46 Y121F T289A mutations that can occur in patients without previous azole exposure have been reported in Europe, Asia, the Middle East, Africa, and Australia. Here, we report the detection of both the TR34 L98H and TR46 Y121F T289A mutations in confirmed *A. fumigatus* isolates collected in institutions in the United States. These mutations, other mutations known to cause azole resistance, and azole MICs are reported here.

Invasive aspergillosis remains a major problem in immunocompromised individuals, including solid organ transplant recipients, those undergoing hematopoietic stem cell transplant, and patients receiving highly immunosuppressive chemotherapies (1–3). In patients with structural damage to the lungs, such as those who have had tuberculosis or sarcoidosis, chronic pulmonary aspergillosis is also a significant problem, the prevalence of which has been estimated to be approximately 3 million patients worldwide (4–7). The azoles are a mainstay in the treatment of invasive aspergillosis, as the members of this class that have activity against *Aspergillus* species, itraconazole, posaconazole, voriconazole, and isavuconazole, may be given orally, and the treatment of this invasive fungal infection is often prolonged. However, prolonged therapy may predispose patients to adverse effects and drug interactions associated with these agents and increase the potential for the development of drug-resistant organisms (8). Over the last several years, concern has been growing regarding azole resistance in *Aspergillus fumigatus* (9, 10). Azole-resistant *A. fumigatus* isolates recovered from patients failing therapy have been reported in several countries around the world. Mutations in the *CYP51A* gene, which encodes the Cyp51 enzyme responsible for the last step in ergosterol biosynthesis, have been the resistance mechanism documented in clinical isolates, and these mutations have been found in isolates recovered from patients who have had long exposures to azoles (11, 12). However, resistant isolates harboring tandem repeats of various sizes in the promoter region of the *CYP51A* gene, along with nonsynonymous point mutations leading to amino acid changes in the Cyp51 enzyme, have been recovered from azole-naive patients with invasive aspergillosis (8, 12, 13). These resistance mechanisms, which include TR34 L98H and TR46 Y121F T289A, have been linked to the environmental use of azoles in agriculture and the preservation of various materials (14, 15), and they have been found in various countries, including many in Europe, India, China, Iran, Tanzania, and Australia (13, 16–21). However, these mutations have not yet been reported in isolates collected in the United States (22). Our objective was to evaluate the Cyp51-associated mechanisms of azole resistance in a collection of *A. fumigatus* isolates from institutions across the United States.

The antifungal susceptibility database in the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio was queried for itraconazole, voriconazole, and posaconazole MIC data against isolates claimed to be *A. fumigatus* from 2001 through 2014. This database is populated with antifungal MIC data against fungal isolates sent to our laboratory from institutions across the United States. Susceptibility testing was performed according to methods in the CLSI M38-A2 standard (23). The MIC was defined as the lowest concentration of each agent that resulted in 100% inhibition of growth after 48 h of incubation at 35°C. Since the CLSI has not established clinical breakpoints against molds, isolates were arbitrarily classified as resistant using the EUCAST breakpoints (voriconazole and itraconazole, ≥4 μg/ml; posaconazole, ≥0.5 μg/ml) (24). Viable isolates with an elevated azole MIC and with a morphology consistent with *A. fumigatus* were subjected to temperature studies at 50°C, and those that grew at this temperature were confirmed to be *A. fumigatus sensu stricto* by sequence analysis of the β-tubulin gene (25, 26). The isavuconazole MICs were also measured against the confirmed *A. fumigatus* isolates. The *CYP51A* gene and its promoter region were also sequenced to evaluate mutations associated with azole resistance. This was done using previously published primers and methods (27–29), and the sequence results were compared to those for the *CYP51A* gene (GenBank accession no. AF338659) (27). If more than one isolate was received from the same patient, only one isolate was included in the analysis.

A total of 220 clinical isolates sent to our laboratory between 2001 and 2014 with an elevated MIC for voriconazole, itraconazole (MIC, ≥4 μg/ml), or posaconazole (≥0.5 μg/ml,) and a preliminary identification of *A. fumigatus* were screened, and 26 nonduplicate isolates with elevated MICs were confirmed to be this

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TABLE 1 UTHSCSA isolate information and MICs[a]

UTHSCSA isolate no.	State[b]	Yr	Cyp51 mutation(s)	MICs for[c]: Itraconazole	Posaconazole	Voriconazole	Isavuconazole
DI15-93	California	2001	M220V	16	1	0.5	1
DI15-95	Connecticut	2007	None detected	>16	2	16	8
DI15-96	Arizona	2008	TR46 Y121F T289A	4	1	>16	>16
DI15-97	North Carolina	2008	None detected	4	1	4	4
DI15-98	Reference laboratory	2008	None detected	16	2	16	16
DI15-99	Maryland	2008	G54R	>16	2	0.5	0.125
DI15-100	Reference laboratory	2008	None detected	>16	1	8	4
DI15-101	Wisconsin	2009	G138S	>16	2	16	>16
DI15-102	Pennsylvania	2010	TR34 L98H	>16	2	8	16
DI15-103	Ohio	2010	M220I	>16	1	0.5	2
DI15-104	Ohio	2011	G448S	4	1	16	8
DI15-105	Reference laboratory	2012	None detected	>16	2	8	8
DI15-106	Reference laboratory	2012	TR46 Y121F T289A	4	1	>16	>16
DI15-107	California	2012	G448S	16	4	0.5	0.25
DI15-108	California	2012	G138C	>16	1	16	8
DI15-109	Washington	2012	G54E	>16	2	0.25	0.125
DI15-110	Connecticut	2013	None detected	>16	2	8	4
DI15-111	Reference laboratory	2013	G54W	>16	>16	0.25	0.125
DI15-112	Ohio	2014	G54R	>16	2	0.25	0.125
DI15-113	Massachusetts	2014	G448S	>16	4	8	4
DI15-114	California	2014	G54R	>16	>16	4	4
DI15-115	Reference laboratory	2014	M220I	>16	1	0.5	1
DI15-116	Pennsylvania	2014	TR34 L98H	>16	1	8	8
DI15-117	Maryland	2014	F219S	>16	2	2	2
DI15-118	Reference laboratory	2014	M220K	>16	4	2	2
DI15-120	Ohio	2015	G448S	>16	1	>16	>16

[a] UTHSCSA, University of Texas Health Science Center at San Antonio.
[b] The states from which the isolates from reference laboratories were obtained are unknown.
[c] The MICs are in μg/ml against 26 *A. fumigatus* isolates.

species by morphology, growth at 50°C, and β-tubulin sequence analysis. Nonsynonymous point mutations in the *CYP51A* gene that result in amino acid changes within the Cyp51 enzyme that are associated with azole resistance in *A. fumigatus* were found in 20 of these isolates, and no point mutations were found in 6 isolates (Table 1). The itraconazole MIC was ≥16 μg/ml against 22 of the 26 isolates, which is consistent with the use of this azole to screen for azole-resistant *A. fumigatus* (14, 21). Two isolates with the TR34 L98H mutations and two with the TR46 Y121F T289A mutations were also found. The mutations were first observed in an isolate from 2008, with two coming from different cities in Pennsylvania, one from Arizona, and one from another reference laboratory. Similar to previous reports, the MICs for voriconazole and isavuconazole were very high (>16 μg/ml) against the two isolates harboring TR46 Y121F T289A mutations, while those of itraconazole and posaconazole were moderately elevated (16, 18). In addition, several point mutations were found that have been associated with the development of azole resistance in patients following long exposures to these agents. The first of these isolates was received by our laboratory in 2001 and came from various states across the United States. The antifungal susceptibility profiles of these isolates varied, which is consistent with previous observations that the location of the point mutation and the corresponding amino acid change can result in different resistance patterns (8). As reported by others, the MIC profile of isavuconazole was similar to that of voriconazole against these isolates (Pearson correlation coefficient, 0.8824; $P < 0.0001$), suggesting that *CYP51A* mutations affect these two antifungals in a similar manner (16, 18). Similar correlations were not observed between posaconazole and voriconazole or isavuconazole (Fig. 1).

To our knowledge, this is the first report of the TR34 L98H and TR46 Y121F T289A mutations in *A. fumigatus* isolates in the United States. A previous study surveillance study of 1,026 clinical *A. fumigatus* isolates collected from various states across the United States did not find these mutations (22). Although the complete patient histories are not known, additional information is available for 3 of these 4 isolates. Each was a clinical isolate (one from tissue, one from bronchoalveolar lavage fluid, and one from sputum) and was collected from patients living in the United States before and around the time the isolates were collected. Two were known to not have travel histories to countries known to harbor these specific mechanisms of resistance, including the one from which the 2008 isolate was collected. Each was also at risk for invasive aspergillosis, and two had confirmed disease, including one with disseminated infection. It is not known if these isolates may have also been recovered from environmental sources, which has been reported in several studies, mainly in Europe, of *A. fumigatus* isolates with these mechanisms of azole resistance (9, 14); however, this has not been a consistent finding (30). Of the 26 azole-resistant isolates included in this study, 6 did not contain mutations within the *CYP51A* gene. Other potential mechanisms of azole resistance that have been reported include higher expression of *CYP51B*, higher expression or modifications in the efflux transporter genes *CDR1B*, *AfuMDR1*, *AfuMDR2*, *AfuMDR3*, and

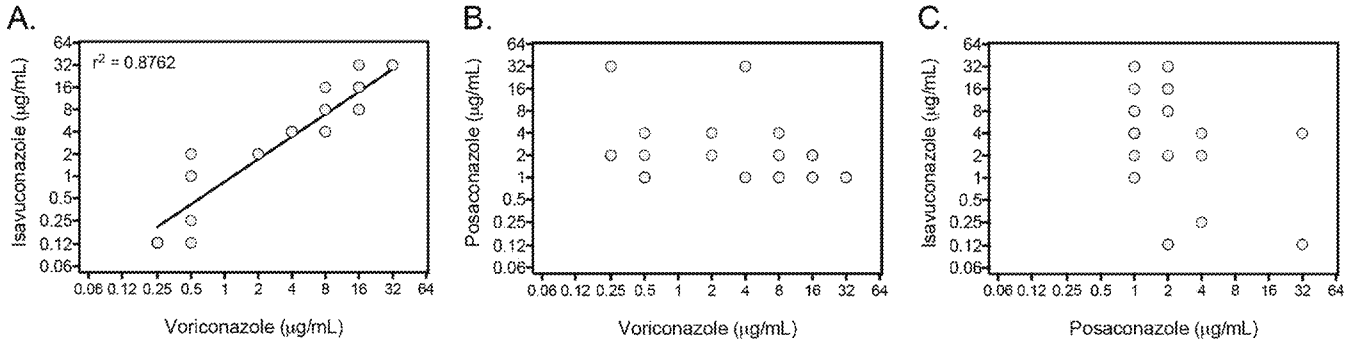

FIG 1 Shown are the MICs for voriconazole versus isavuconazole (A), voriconazole versus posaconazole (B), and posaconazole versus isavuconazole (C). All MICs were read as 100% inhibition of growth after 48 h of incubation at 35°C. MICs of >16 μg/ml were plotted at 32 μg/ml.

AfuMDR4, and a mutation in the CCAAT-binding transcript factor complex subunit HapE (31–35). However, these mechanisms of resistance were not evaluated in this study, and their clinical relevance is unknown.

Our findings demonstrate that the TR34 L98H and TR46 Y121F T289A mutations can be found in the United States and that there is a need for continued surveillance of azole resistance in *A. fumigatus*.

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